



PII: S0045-6535(98)00318-X

IDENTIFICATION OF A MULTIXENOBIOTIC RESISTANCE MECHANISM IN *XENOPUS LAEVIS* EMBRYOS

P. Bonfanti, A. Colombo and M. Camatini

Dipartimento di Scienze dell'Ambiente e del Territorio, Università degli Studi di Milano, via Emanuelli 15, 20126 Milano, Italy.

ABSTRACT

In the '90's a membrane-associated transport protein, discovered in aquatic organisms, was considered to be expressed in response to environmental xenobiotics. Like the multidrug resistance protein found in mammalian tumor cell lines, this protein confers resistance in organisms in polluted areas by binding xenobiotics and transporting them out of the cells in an energy-dependent manner.

This study investigates the expression and the activity of a P-glycoprotein (Pgp) involved in a multixenobiotic resistance mechanism (MXRM) during the early developmental stages and in tissues of adult *Xenopus laevis*. ©1998 Elsevier Science Ltd. All rights reserved

KEY WORDS: multixenobiotic resistance mechanism (MXRM), *Xenopus laevis*, P-glycoprotein (Pgp).

INTRODUCTION

In response to pollutants, aquatic organisms demonstrate several defence mechanisms, such as the induction of detoxification enzymatic systems. Among these cytochrome P-450 [1], glutathione transferase [2], ethoxyresorufin-O-deethylase (EROD) [3], metallothioneines [4], are involved in transformation and conjugation of these compounds.

Recently, a P-glycoprotein (Pgp) mediated multixenobiotic resistance mechanism (MXRM), discovered in marine sponges [5] and also found in mussels and fish [6; 7; 8], was considered to be expressed as a biological defence mechanism against environmental xenobiotics.

This protein belongs to a large family of high molecular weight membrane glycoproteins that can confer a multidrug resistance (MDR) phenotype, well characterised in mammalian tumor cell lines [9; 10; 11].

This Pgp acts as a cell-surface pump that confers MDR to cancer cells by maintaining intracellular levels of a variety of structurally and functionally unrelated cytotoxic agents below a killing threshold [9]. A common feature among substrates of the transporter appears to be a moderate hydrophobicity and a positively charge domain [12].

The protein shows internal homology between its amino (N)- and carboxy (C)-terminal halves, each with 6 putative transmembrane helices and a consensus ATP binding site [10].

Some drugs, like verapamil, are known to reverse the MDR phenotype in mammalian systems by binding to the active site of Pgp, causing an inhibition of efflux of cytotoxic drugs and hence restoring the previous sensitivity to them [13].

The aim of this paper was to use Western blot analysis to identify the presence of a protein immunochemically related to the mammalian transporter in adult and embryos of *Xenopus laevis*. With a fluorescence dye transport assay we discovered that the transport activity of this protein in embryos is modulated by verapamil as well as the mammalian MDR Pgp and the aquatic organism multi xenobiotic resistance (MXR) Pgp.

MATERIALS AND METHODS

Chemicals and antibodies

Rhodamine B Amine, anti-goat IgG alkaline phosphatase-conjugate and all other chemicals were obtained from Sigma-Aldrich srl, (Milan, Italy) and monoclonal anti-P-Glycoprotein 170-180 from mouse-mouse hybrid cells (clone JSB-1) from Boehringer Mannheim Biochemica (Milan, Italy).

Animals

Fresh-water *Anodonta cignea* mussels, were collected from the Varese lake, Italy, and maintained in flowing fresh-water aquariums until used for laboratory experiments.

Xenopus laevis adults were purchased from Dr. Schneider (Varese, Italy).

Xenopus laevis embryos were obtained by *in vitro* fertilisation according to Colombo et al. [14].

Immunocytochemistry

For Western blot analyses, gill tissues from *Anodonta cingea*, *Xenopus laevis* embryos (stage 51) and liver, intestine, lung, testis, ovary tissues from adult *Xenopus* were rinsed in 0.9% NaCl with 1mM phenylmethylsulfonyl fluoride (PMSF), chopped and sonicated for 20 s (Labsonic L B Braun 1000) in lysis buffer 3 v/w (10mM KCl, 1.5mM MgCl₂, 10mM Tris-HCl, 2mM PMSF and 5% sodium dodecyl sulphate (SDS), pH 7.4). The samples were then centrifuged for 10 min at 10,000 g. The pellets were discarded and protein concentration determined in the supernatants by Lowry assay [15].

40µg of the SDS-solubilized sample proteins were resolved on 7.5% gels by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli [16], transferred to nitrocellulose [17] and incubated first with the monoclonal antibody (1µg/ml). The immune complexes formed were visualised after additional incubation with anti-mouse IgG alkaline phosphatase-conjugated, applying the nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates of phosphatase reaction.

Multixenobiotic transport activity

The *Xenopus laevis* MXR activity was investigated at morula stage (3.5 h after fertilisation) and in embryos at stage 22 (24 h after fertilization). Protein activated exclusion of the fluorescence rhodamine dye from the cells was observed.

Briefly, groups of 10 embryos were incubated for 1h with 1µM rhodamine in the presence or absence of 22µM verapamil, an inhibitor substrate of mammalian MDR activity. At the end of the incubation period (1h) the embryos were quickly washed free of the drug with a physiological solution (NaCl 10.6mM, NaHCO₃ 1.14 mM, KCl 0.4mM, CaCl₂ 2H₂O 0.087mM, CaSO₄ 2H₂O 0.408mM, MgSO₄ 0.622mM), placed in Petri dishes and observed with a 5X lens on a Zeiss fluorescence microscope (Axioplan) equipped with a camera.

Rhodamine accumulation was measured in stage 22 embryos by spectrofluorimetric techniques (Jasco FP-777). The embryos were incubated in 200µM rhodamine or in rhodamine plus 22µM verapamil and the fluorescence of 10 embryos was measured at various intervals over 1h and 30 min period after sonication.

RESULTS

A protein related to the mammalian multidrug transporter was detected in protein membrane preparations of *Anodonta cingnea* and *Xenopus laevis* samples (Fig. 1a).

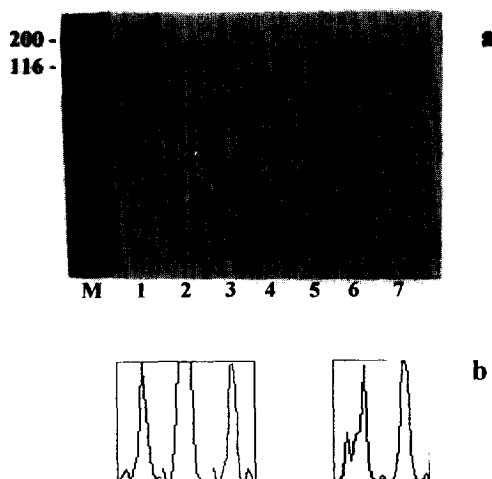


Fig. 1 Detection of the multixenobiotic transport protein in *Xenopus laevis*. **a)** Western blot of membrane protein extracts from *Xenopus* adult tissues and embryos. All bands were derived from 40µg of membrane protein and detected with the JSB-1 antibody. Lane M: molecular weight standards; lane 1: *Anodonta cingnea* gills used as a positive control; lane 2: *Xenopus* liver; lane 3: *Xenopus* intestine; lane 4: *Xenopus* lung; lane 5: *Xenopus* ovary; lane 6: *Xenopus* testis; lane 7: *Xenopus* embryos (stage 51). **b)** Computer image processing of immunorecognised proteins.

The monoclonal antibody directed toward a conserved epitope of the membrane associated internal domain of the P-glycoprotein 170-180 kDa recognised a single protein band with an apparent molecular weight between 116 and 205 kDa in *Anodonta cingnea* gills used as a positive control (Fig. 1a, lane 1), in *Xenopus laevis* embryos (Fig. 1a, lane 7) and in liver (Fig. 1a, lane 2), intestine (Fig. 1a, lane 3) and testis (Fig. 1a, lane 6) of adult *Xenopus*. This antibody did not label any protein in lung and ovary (Fig. 1a, lanes 4 and 5). The computer analysis of the colour intensity associated with the bands revealed peaks higher in liver and embryos than in other samples (Fig. 1b).

At morula stage and stage 22 exposure of *Xenopus* embryos to rhodamine dye, a substrate of the mammalian multidrug transporter, was used to demonstrate the multixenobiotic transport activity. When the embryos were exposed to rhodamine alone, the fluorescence remained low (Figs. 2a, 3a); however embryos in rhodamine plus verapamil accumulated much more dye, which showed the inhibition of the transporter (Figs. 2b, 3b).

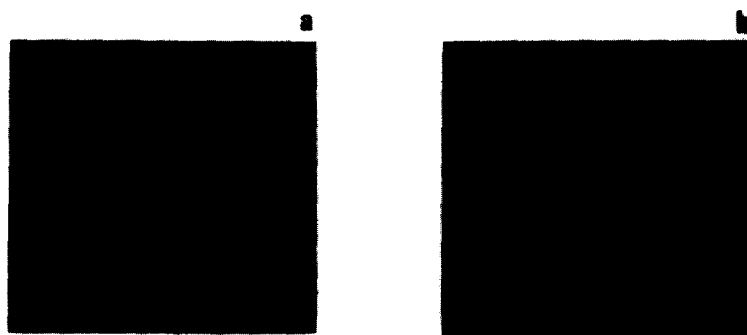


Fig. 2 Morula stage *Xenopus* embryos. **a)** incubation in 1 μ M rhodamine. **b)** incubation in 1 μ M rhodamine plus 22 μ M verapamil.

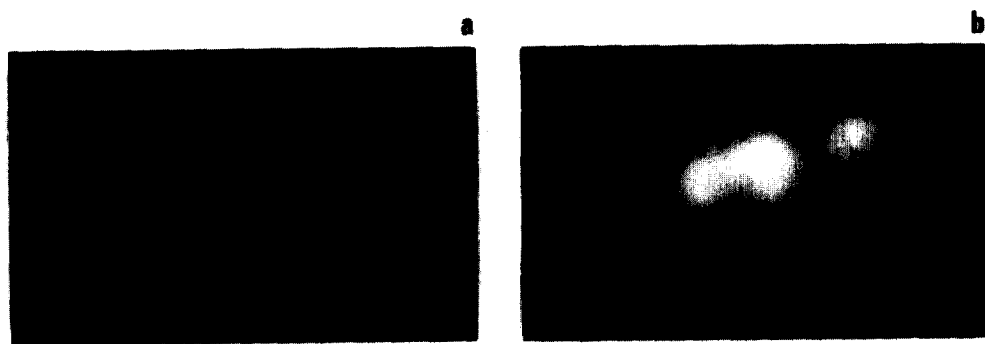


Fig. 3 Stage 22 *Xenopus* embryos. **a)** incubation in 1 μ M rhodamine. **b)** 1 μ M rhodamine plus 22 μ M verapamil.

This different rhodamine accumulation is much more evident in stage 22 (Fig. 3) than in morula stage embryos (Fig. 2); in particular in stage 22 embryos incubated in verapamil, rhodamine was distributed in three specific spots along the cephalic-caudal axis (Fig. 3b).

In addition to quantify dye transport activity, fluorescence was measured at different times by spectrofluorimetric techniques over a 90 min period. The presence of verapamil enhanced the accumulation of dye over rhodamine embryos values, even if the shape of the curves was almost the same (Fig. 4).

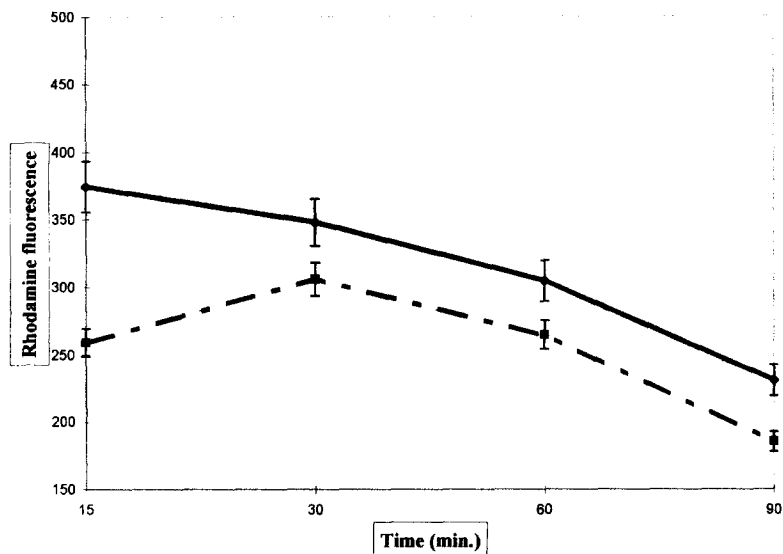


Fig. 4 Time course of rhodamine accumulation on stage 22 *Xenopus* embryos incubated in 200µM rhodamine (• — •) and 200µM rhodamine plus 22µM verapamil (—). The fluorescence was measured at various times with a spectrofluorimeter.

DISCUSSION

Our earlier studies demonstrated that the amphibian *Xenopus laevis* expresses detoxification enzymatic systems such as cytochrome P-450 [14] and glutathione transferase [18]. Now we have found a MXRM which is supposed to be the first line of defence against many lipophilic xenobiotics in several aquatic organisms [8].

Applying a monoclonal antibody raised against an highly conserved epitope of Pgp one protein band was visualised in the positive control *Anodonta cygnea* as well as in *Xenopus laevis*. The specificity of this antibody indicates that this protein is related by sequence to the mammalian Pgp. Furthermore the size of the protein falls within the range of molecular weights (120-200 KDa) reported for Pgp in relation to its

mobility on gel electrophoresis [10]. The high sequence conservation of Pgp during species evolution suggests that its function is of fundamental importance as it is a common route for the export of toxic compounds.

The immunoreactive proteins, expressed both in adult and embryos of *Xenopus laevis*, show very little different molecular weights as reported by Toomey and Epel [19] in *Urechis caupo*. The protein seems to be localised in specific tissues of the adult frogs such as liver, intestine and testis as well as in mammals [20; 10]. Many other authors have supposed that the Pgp in these specific locations is a factor which limits intestinal absorption, testis diffusion as well as a feature which participates in liver in the excretion of xenobiotics and their metabolites into bile [21; 20].

No detectable Pgp levels were revealed in the membrane of *Xenopus* lung, while in mammals it is recognisable in some cases of untreated lung cancer [22]. The absence of immunoreaction was obtained also in *Xenopus* ovary. This result has to be confirmed by future analysis, because the method used to extract membrane proteins did not eliminate the yolk polypeptides that can cause trouble when preparing samples for SDS-PAGE and immunoblotting as these proteins can often nonspecifically react with antibodies or mask a signal [23].

High levels of Pgp were also found in embryos (stage 51) which suggests its important role in protecting this organism against xenobiotic exposure during this sensitive step in its life.

To support the immunochemical hypothesis, a functional analysis was performed on morula and stages 22 *Xenopus*, by measuring the sensitivity of rodhamine dye accumulation to verapamil. This accumulation assay showed that inhibitors of the mammalian multidrug transporter, such as verapamil, also inhibit dye accumulation in aquatic organisms, implying the existence of this type of transport activity in organisms other than mammals [24; 25; 26]. Our results demonstrate that in the *Xenopus* rhodamine accumulated intracellularly to a greater extent in the presence of verapamil, which shows a modulation of Pgp-mediated rodhamine export from the cells similar to that found in mammals and other aquatic organisms.

These data strongly suggest the existence of a Pgp immunologically related to the mammalian multidrug transport protein both in embryos and adults of the *Xenopus laevis*, which acts as a first line of defence by decreasing the accumulation of xenobiotics in the cells.

Further studies performed on *Xenopus laevis* embryos exposed to lipophilic xenobiotics would provide a correlation between the level of these pollutants and the protein expression to use Pgp as a valuable molecular indicator.

Acknowledgements

This research was supported by a grant from National Research Council N. 96.00797CT13 to M.C.

REFERENCES

1. J.J. Stegeman, B.R. Woodin and A. Goksoyr, Apparent cytochrome P-450 induction as an indication of exposure to environmental chemicals in the flounder *Platichthys flesus*, *Mar. Ecol. Prog. Ser.* 46, 55-60 (1988).
2. J. Harris, B. Coles, D.J. Meyer and B. Ketterer, The isolation and characterization of the major glutathione S-transferase from the squid *Loligo vulgaris*, *Comp. Biochem. Physiol.* 98B, 511-515 (1991).
3. D. Grzebyk and F. Galgani, Measurement of the effect of organic pollution on marine organisms. Rapid determination of EROD induction using plate readers, *Aquat. Living. Resour.* 4, 53-59 (1991).
4. P.E. Olsson and C. Hogstrand, Subcellular distribution and binding of cadmium to metallothionein in tissues of rainbow trout, *Environ. Toxicol. Chem.* 6, 867-874 (1987).
5. Kurelec and B. Pivcevic, Distinct glutathione-dependent enzyme activities and a verapamil-sensitive binding of xenobiotics in a fresh-water mussel *Anodonta cygnea*, *Biochem. Biophys. Res. Commun.* 164, 934-940 (1989).
6. B. Kurelec and B. Pivcevic, Evidence for a multi-xenobiotic resistance mechanism in a mussel *Mytilus galloprovincialis*, *Aquat. Toxicol.* 19, 291-302 (1991).
7. C. Minier, F. Akcha and F. Galgani, P-glycoprotein expression in *Crassostrea gigas* and *Mytilus edulis* in polluted seawater, *Comp. Biochem. Physiol.* 106B, 1029-1036 (1993).
8. B. Kurelec, Inhibition of multixenobiotic resistance mechanism in aquatic organisms: ecotoxic consequences, *Sci. Total Envir.* 171, 197-204 (1995).
9. R.L. Juliano and V. Ling, A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants, *Biochim. Biophys. Acta* 455, 152-162 (1976).
10. J.A. Endicott and V. Ling, The biochemistry of P-glycoprotein-mediated multidrug resistance, *Annu. Rev. Biochem.* 58, 137-171 (1989).
11. A.H. Schinkel and P. Borst, Multidrug resistance mediated by P-glycoproteins, *Cancer Biol.* 2, 213-226 (1991).

12. H.L Pearce, A.R. Safa, N.J. Bach, M.A. Winter, M.C. Cirtain and W.T. Beck, Essential features of the P-glycoprotein pharmacophore as defined by a series of reserpine analogs that modulate multidrug resistance, *Proc. Nat. Acad. Sci. U.S.A.* 86, 5128-5132.
13. K.Yusa and T. Tsuru, Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outwards across the plasma membrane of K562/ADM cells, *Cancer Res.* 49, 5002-5006.
14. A. Colombo, P. Bonfanti, M. Ciccotelli, M. Doldi, N. Dell'Orto and M. Camatini, Induction of cytochrome P4501A isoform in *Xenopus laevis* is a valid tool for monitoring the exposure to benzo[a]pyrene, *J. Aquat. Ecosystem Health*, 5, 207-211 (1996).
15. O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193, 265-275 (1951).
16. U.K. Laemmli, Cleavage of structural proteins during assembly of the head of the bacteriophage T4, *Nature* 227, 680-685 (1970).
17. W.N. Burnette, Western blotting: electrophoretic transfer of protein from sodium dodecyl sulphate-polyacrilamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, *Anal. Biochem.* 112, 195-203 (1981).
18. M. Ciccotelli, S. Crippa and A. Colombo, Bioindicators for toxicity on effluents from wastewater treatment plants, International symposium on integrated ecotoxicology from molecules/organisms to ecosystems, Milan, Italy (June, 1987).
19. B.H. Toomey and D. Epel, Multixenobiotic resistance in *Urechis caupo* embryos: protection from environmental toxins, *Bio. Bull.* 185, 355-364 (1993).
20. T.W. Gant, C.K. O'Connor, R. Corbitt, U. Thorgeirsson and S.S. Thorgeirsson, *In vivo* induction of liver P-glycoprotein expression by xenobiotics in monkeys, *Toxicol. Appl. Pharmacol.* 133, 269-276 (1995).
21. D. Levêque and F. Jehl, P-glycoprotein and pharmacokinetics, *Anticanc. Res.* 15, 331-336 (1995).
22. I. Sugawara, I. Kataoka, Y. Morishita, H. Hamada, T. Tsuruo, S. Itoyama and S. Mori, Tissue distribution of P-glycoprotein encoded by a multidrug-resistance gene as revealed by a monoclonal antibody, MRK 16, *Canc. Res.* 48, 1926-1929 (1988).

23. P.E. Janice and K.K. Brian. Biochemical fractionation of oocytes, p. 133. Academic Press, London (1991).
24. B Kurelec, S. Krca, B. Pivcevic, D. Ugarkovic, M. Bachmann, G. Imsiecke and W. E.G. Muller, Expression of P-glycoprotein gene in marine sponges. Identification and characterization of the 125 kDa drug-binding glycoprotein, *Carcinogenesis* 13, 69-76 (1992).
25. B Kurelec, P. Waldmann and R.K. Zahn, The modulation of the protective effects of the multixenobiotic resistance mechanism in a clam *Corbicula fluminea*, *Mar. Environ. Res.* 42, 383-387 (1996).
26. F. Galgani, R. Cornwall, B.H. Toomey and D.D Epel, Interaction of environmental xenobiotics with a multixenobiotic defense mechanism in the bay mussel *Mytilus galloprovincialis* from the coast of California, *Environ. Toxicol. Chem.* 15, 325-331 (1996).